Limited Role for Iron Regulation in *Coxiella burnetii* Pathogenesis

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Received 5 December 2007/Returned for modification 9 January 2008/Accepted 20 February 2008

In gram-negative bacteria, iron acquisition proteins are commonly regulated by Fur (ferric uptake regulator), which binds iron-regulated promoters (the Fur box). We hypothesized that *Coxiella burnetii* requires iron and employs an iron-regulatory system and used various approaches to define a Fur regulon. Cloned *C. burnetii fur* complemented an *Escherichia coli fur* deletion mutant. A ferrous iron transporter gene (CBU1766), a putative iron binding protein-encoding gene (CBU0970), and a cation efflux pump gene (CBU1362) were identified by genome annotation and using a Fur titration assay. Bioinformatically predicted Fur box-containing promoters were tested for transcriptional control by iron. Five genes demonstrated at least a twofold induction with minimal iron. Putatively regulated genes were evaluated in a two-plasmid regulator/promoter heterologous expression system. These data suggested a very limited Fur-regulated system in *C. burnetii*. In an in vitro tissue culture model, a significant increase in bacterial growth was observed with infected cells treated with deferoxamine in comparison to growth under iron-replete conditions. In an iron-overloaded animal model in vivo, the level of bacterial growth detected in the iron-injected animals was significantly decreased in comparison to growth in control animals. In a low-iron-diet animal model, a significant increase in splenomegaly was observed, but no significant change in bacterial growth was identified. The small number of predicted iron acquisition systems, few Fur-regulated genes, and enhanced replication under a decreased iron level predict a requirement of a low level of iron for survival, perhaps to avoid creation of additional reactive oxygen radicals.

*Coxiella burnetii*, the etiologic agent of Q fever, is an obligate, intracellular, gram-negative bacterium. In vitro, it can replicate in a range of cell lines, including fibroblasts and macrophage-like cells (28). Transmission of bacteria occurs primarily by the aerosol route. Patients with acute Q fever typically present with fatigue, high fever, and periobital headache, while chronic Q fever usually develops as endocarditis or hepatitis (42). The bacterium is found in a variety of animal reservoirs, such as arthropods, sheep, goats, and cattle (28). Therefore, Q fever is considered an occupational hazard for those in close contact with livestock (40). The Centers for Disease Control and Prevention have classified *C. burnetii* as a class B bioterrorism agent (13).

The ability to acquire iron is essential in the pathogenesis of the majority of infectious bacteria (4). Iron is used in functions as diverse as respiration, oxygen transport, and DNA biosynthesis (4). However, acquisition must be tightly regulated, since excess iron results in the generation of oxygen radicals via the Fenton and Haber-Weiss reactions. These oxygen radicals can cause damage in DNA and other critical macromolecular structures (60). Expression of iron acquisition proteins in bacteria is typically transcriptionally regulated by a repressor, described as the ferric uptake regulator (Fur) in *Escherichia coli* (26). Homologues in gram-positive and gram-negative bacteria, including phylogenetic relatives of *Coxiella*, the intracellular pathogenic *Legionella pneumophila*, have been described previously (4, 30). The prototypic model for transcriptional regulation is, in an iron-replete environment, Fur binding its corepressor, Fe²⁺. The complex blocks transcription of Fur-regulated genes by binding to a palindromic sequence, which is known as a Fur box, present in the promoter region (19). This typically results in transcriptional repression. A few experimental models have noted activation of some promoters (17, 20). Masse et al. recently described a process of Fur activation via an indirect method involving a small RNA, the RyhB RNA (41).

*C. burnetii* replicates within a vacuole with properties similar to the phagolysosome of host cells, normally an inhospitable environment (8). It is currently unknown what nutrients are available in this milieu. Howe and Mallavia reported an up-regulation of transferrin receptor expression during the first 24 h of infection, suggesting a correlation between iron acquisition and infection. The authors also predicted an absolute requirement for iron by describing inhibition of *C. burnetii* replication in J774A.1 murine macrophage-like cells incubated in the presence of deferoxamine, an iron chelator (33). We hypothesized that, similar to *Legionella* spp., *C. burnetii* employs a functional Fur regulon to maintain essential levels of iron.

In this paper, we describe the characterization of a *C. burnetii fur* homologue, which functionally complements an *E. coli fur* deletion strain. We identify a putative Fur-regulated gene (frg1) and a cation efflux pump (CBU1362) by using a Fur titration assay (FURTA) and an iron acquisition gene homologue, the ferrous iron transporter *feoB*, by analyzing the gene...
nome annotation of C. burnetii. Previous, putative members of a Fur regulon were identified by bioinformatic screening of promoter regions for all C. burnetii open reading frames (ORFs). (C. burnetii ORFs identified by the CBU numbers presented in the text were assigned locus tags in The Institute for Genomic Research [TIGR] annotation [55].) Members of the putative Fur regulon were evaluated by transcriptional profiling of C. burnetii in axenic acid activation buffer and by a two-plasmid regulator/promoter heterologous system. We also predict a requirement of low levels of iron for C. burnetii replication in both tissue culture and animal models of infection. We conclude that C. burnetii likely encodes a limited set of iron transport systems to prevent oxidative damage associated with iron acquisition.

MATERIALS AND METHODS

Media and chemicals. Luria-Bertani (LB) medium (Difco, Detroit, MI) was prepared according to a laboratory manual (39). Antibiotics were incorporated into media at the following concentrations to maintain plasmids in E. coli: kanamycin, 50 μg ml−1; tetracycline, 12.5 μg ml−1; and chloramphenicol, 30 μg ml−1. Purified C. burnetii was incubated at 37°C for 24 h in an acid activation buffer. The buffer (32 mM KPO4, 15 mM NaCl, 152.2 mM KCl, 100 mM kanamycin, 50 μg ml−1) was centrifuged at 4°C for 10 min. The supernatant was centrifuged at 13,600 g for 30 min. The pellets were resuspended in 0.25 M sucrose phosphate buffer (53.9 mM Na2HPO4, 12.8 mM KH2PO4, 72.6 mM NaCl, 0.25 M sucrose). The solution was centrifuged at 9000 g for 20 min. The supernatant was centrifuged at 13,600 g for 20 min, and the resulting pellet was resuspended in 0.25 M sucrose phosphate buffer (53.9 mM Na2HPO4, 12.8 mM KH2PO4, 72.6 mM NaCl, 0.25 M sucrose). The solution was centrifuged at 13,600 g for 30 min to remove cellular debris. These two spins were alternated until minimal amounts of debris were detected. Bacteria were stored in 0.25 M sucrose phosphate buffer.

Isolation of DNA. Plasmid minipreps were prepared by using the FastPlasmid miniprep kit described as the manufacturer (Eppendorf, Hamburg, Germany). The protocol for extraction of C. burnetii Nine Mile DNA from infected cells was as follows: Infected cells were resuspended in lysostaphin (0.25 M Tris pH 7.5, 10 mM EDTA, 5 mM glucose, 200 μg lysosome per ml solution) with 1 mg of protease K. Cells were incubated at 60°C for 4 h. Sodium dodecyl sulfate (SDS) was added at a final concentration of 1% and incubated at room temperature for 1 h. The protocol from High Pure PCR template preparation kit was performed as described by the manufacturer (Roche Molecular Biochemicals, Indianapolis, IN).

Plasmid construction. For the construction of pNP101 and pNP104, the C. burnetii Fur sequence was cloned into pCR 2.1/TOPO (Invitrogen) by using the primers ChFur-For (CGGTAGGTTGGACGCGC) and ChFur-Rev (TGGACTTAAGGTTTAAAG) and designated pNP101. ChNP104 was then digested with EcoRI (New England Biolabs, Ipswich, MA), resulting in an 820-bp fragment, then cloned into pACYC184, and the construct was designated pNP104.

Primers for the two plasmid system (see Table SI in the supplemental material) were used at a final concentration of 1 μM per 50-μl reaction volume. The amplification procedure consisted of 94°C for 1 min, 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min, and 72°C for 2 min with a 4-s pause. Primers (Integrated DNA Technologies, Inc., Coralville, IA) were used as described in the supplemental material and C. burnetii RSA 493 (Nine Mile phase I) DNA were mixed with PCR SuperMix (Invitrogen, Carlsbad, CA), and reactions were carried out using the DNA Engine PT-200 Polich thermal cycler (MJ Research, Watertown, MA). PCR products were subsequently cloned into PCR cloning vector pBlue-TOPO (Invitrogen, Carlsbad, CA).

Real-time PCR. Standards were generated by 10-fold serial dilutions of 107 to 1 organisms. The substrate for LacZ hydrolysis in this assay was 59°C for 1 min, and 72°C for 7 min, and 72°C for 6 min with a 4-s pause. Primers (Integrated DNA Technologies, Inc., Coralville, IA) were used as described in the supplemental material and C. burnetii RSA 493 (Nine Mile phase I) DNA were mixed with PCR SuperMix (Invitrogen, Carlsbad, CA), and reactions were carried out using the DNA Engine PT-200 Polich thermal cycler (MJ Research, Watertown, MA). PCR products were subsequently cloned into PCR cloning vector pBlue-TOPO (Invitrogen, Carlsbad, CA).

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**FIG. 1.** ClustalW alignment of Fur homologues. Amino acid alignment of *C. burnetii* Fur with Fur homologues from *E. coli* K-12 and *L. pneumophila* Paris. The shaded areas are similarity matches among the species. The area underlined is the HHXHX2CX2C motif (47).

*Legionella pneumophila*-infected J774A.1 cell experiment. *L. pneumophila* was grown on buffered charcoal-yeast extract agar plates between 48 and 72 h. Tissue culture cells were plated at 5 × 10⁵ cells/ml in 24-well tissue culture plates and incubated overnight at 37°C in 5% CO₂. Twenty-four hours after tissue culture seeding, bacteria were swabbed off the agar plate into buffered yeast extract broth. The optical density at 600 nm was measured, and J774A.1 cells were infected with *L. pneumophila* at a multiplicity of infection of 1:10. The infected cells were incubated for 1 h at 37°C in 5% CO₂. After incubation, the cells were washed with prewarmed phosphate-buffered saline, and fresh medium with 0 μM, 30 μM, or 50 μM deferoxamine was added. The cells were lysed in 1 ml of distilled water at 0 h, 24 h, and 48 h after deferoxamine treatment. The bacteria were centrifuged and the pellet was resuspended in buffered yeast extract broth. The optical density at 600 nm was measured, and J774A.1 cells were grown on buffered charcoal-yeast extract agar plates between 48 and 72 h.

*C. burnetii* infection in BALB/c mice. Forty-eight female, six-week-old BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were divided into four groups of six mice each for two studies, utilizing iron deficiency and iron overload. Mice in group I served as negative controls with no experimental manipulation. Group II mice were fed an iron-deficient custom research diet (TD 80396; Harlan Teklad, Indianapolis, IN) ad libitum and were given deionized drinking water for two weeks prior to infection and throughout infection. Group III mice were also provided an iron deficiency diet but were injected intraperitoneally (i.p.) with 0.5 ml deferoxamine every other day for two weeks prior to infection and throughout infection. Group III mice were negative controls and received sterile saline injections intraperitoneally (i.p.) every other day for 2 weeks. Mice in group IV were iron overload by i.p. injection of 1.2 mg iron dextran (Vedco, St. Joseph, MO) every other day for two weeks prior to infection and throughout infection. Animals in groups I, III, and IV were provided a commercial rodent diet (Harlan Teklad, Indianapolis, IN) and water ad libitum.

All mice were infected i.p. with 10⁷ phase 1 *C. burnetii* Nine Mile (RSA493) cells suspended in phosphate-buffered saline. Body weights were measured prechallenge and 14 days postchallenge. Animals in all groups were euthanized on day 14 postinfection by carbon dioxide overdose following exsanguination, and spleen weights were measured. Liver, spleen, and bone were collected at necropsy for histopathologic evaluation. Serum samples were collected for measurement of iron. All animal experimentation was reviewed and approved by Texas A&M University Laboratory Animal Care Committee and was performed in compliance with university and federal regulations. All animal experiments were conducted in a biosafety level III laboratory.

Quantification of *C. burnetii* in spleens. Approximately 0.025 g of spleen from each infected animal was homogenized in 500 μl lysis buffer (6.25 ml 2 M Tris, 1 ml 0.5 M EDTA, 50 mg glucose, 200 mg lysozyme). Homogenate (200 μl) was then incubated with 10 μl proteinase K for 4 h at 60°C. The lysate was then incubated with 21 μl 10% SDS at room temperature for 1 h. The High Pure PCR template preparation kit (Roche, Indianapolis, IN) was used to extract DNA, per the instructions of the manufacturer, following tissue lysis as previously described (2). *C. burnetii* genome copies were enumerated by real-time PCR using primers amplifying the com1 gene as previously reported (10). Measurement of serum iron. At day 14 postinfection, serum samples were collected. Serum iron levels were determined using the iron/total iron-binding capacity reagent set according to the manufacturer’s protocol (Pointe Scientific, Canton, MI).

Calculation of bacterium generation time. G = e[(3.3 × log (b/B)], where G is the generation time, t is the time interval (in hours), b is the genome copy number at the end of the interval, and B is the genome copy number at the start of the interval (http://textbookofbacteriology.net/growth.html).

**RESULTS**

Identification of *C. burnetii fur*. Since Howe and Mallavia suggested an absolute iron requirement for *C. burnetii* pathogenesis (33), we hypothesized that a Fur homologue may have a role in regulation of iron acquisition in *C. burnetii*. Analysis of the TIGR annotation of the *C. burnetii* Nine Mile genome revealed the nucleotide sequence for *fur* (CBU1301) (55). BLAST alignment of the annotated amino acid sequence of *C. burnetii* Fur showed 58% identity and 77% similarity to the *E. coli* K-12 Fur protein (accession number NP_415209) and 71% identity and 84% similarity to Fur from *L. pneumophila* (accession number CAH11586) (Fig. 1). Unlike the case for *fur*...
homologues in other organisms, sequence analysis of the predicted promoter region did not predict a Fur box; therefore, autoregulation by Fur was not expected.

**C. burnetii Fur complementation of an E. coli fur mutant.** Since the ability to generate a C. burnetii mutant is currently unavailable, the functionality of C. burnetii Fur was demonstrated by complementing an E. coli fur deletion strain containing a *fhuF::lacZ* reporter fusion (RRJC-1) (Table 1). *fhuF* is a Fur-regulated gene in *E. coli*, thus, heterologous Fur proteins can be evaluated. Promoter activity was assessed quantitatively (43).

The complemented strain containing C. burnetii fur (pNP101) in the presence of iron showed a significant decrease in *β*-galactosidase activity compared to the *β*-galactosidase activity of uncomplemented fur mutant (RRJC-1) (*P* = 0.001) (Fig. 2). To confirm iron as the regulatory cofactor in the complemented strain, 200 μM deferoxamine, an iron chelator, was added to the cultures. Under these conditions, derepression of *fhuF* was observed by a significant increase in *β*-galactosidase activity compared to that in the iron-containing samples (*P* < 0.01) (Fig. 2). As a positive control, the E. coli fur gene, carried on pMH15 (Table 1), was also transformed into RRJC-1, and a decrease in *β*-galactosidase activity in the presence of iron (*P* < 0.01) (Fig. 2) was observed.

**Identification of Fur-regulated genes in the FURTA.** Since C. burnetii fur complemented an E. coli fur deletion mutant, we then identified putative C. burnetii Fur-regulated genes. Initially, we used the FURTA with the E. coli strain (H1717). The FURTA uses the binding of the Fur-iron complex to the palindromic sequence (Fur box) in the promoter region to identify Fur-regulated genes in *E. coli* (57). Since E. coli Fur has shown the ability to bind to Fur boxes in other organisms, the FURTA has been successfully used to identify Fur-regulated genes of several bacteria (7, 21, 27). The FURTA reporter E. coli strain H1717 contains a chromosomal copy of *E. coli* fur and a *fhuF::lacZ* reporter fusion. Two independently generated C.

![FIG. 2. C. burnetii fur complements an E. coli fur deletion mutant. C. burnetii fur(pNP101) was transformed into an E. coli fur mutant. Cultures were grown in the presence of iron or the iron chelator deferoxamine (200 μM). The *fhuF::lacZ* reporter fusion activity is reported as Miller units. C. burnetii Fur complemented the mutant and showed a significant decrease in activity in the presence of iron (the asterisk indicates a *P* value of <0.01). Deregulation of C. burnetii Fur was observed in the iron-depleted samples (the asterisk indicates a *P* value of <0.01).]
burnetii restriction fragment libraries were transformed into H1717. Plasmid inserts containing putative Fur boxes were identified by a derepression of the fluF::lacZ fusion in the presence of iron (50 μM FeCl₃), as Fur preferentially bound to the numerous copies of the insert.

Unexpectedly, only two genes were identified in this assay (CBU0970 and CBU1362), although over 20,000 clones were screened from two independently generated libraries. We designated CBU0970 frg1 (fur-regulated gene 1). With the TIGR Nine Mile annotated sequence, a BLAST analysis did not reveal any significant homology for frg1 to previously characterized genes in the database. Using SignalP-HMM amino acid sequence analysis, a signal sequence was predicted between M1 and A26 (45, 46). Amino acid sequence analysis also identified a histidine-rich region at H52 to H60 and two possible metal binding sites, C118 to C123 (CX₄C) and C260 to C264 (CX₄C). Sequence analysis also revealed a putative Fur box in the promoter region with 74% identity to the E. coli Fur box consensus sequence (Fig. 3) (57).

BLAST analysis of the CBU1362 annotated sequence predicted similarity to a family of cation efflux proteins. Members of this family belong to the larger group of cation diffusion facilitator (CDF) proteins and are primarily involved in metal ion transport (5). ClustalW sequence analysis of the promoter region predicted a region with 58% identity to the E. coli Fur box consensus sequence (Fig. 3) (57).

Survey of the C. burnetii Nine Mile annotated genome. We recently reported the identification of feoB (CBU1766), a ferrous iron transporter gene, in the genome annotation and the initial characterization of C. burnetii Fur regulation of the gene (11). FeoB has been previously described as a Fur-regulated ferrous iron acquisition protein in E. coli (34). A BLASTP alignment of the predicted amino acid sequence of C. burnetii FeoB indicated that it was 47% identical and 66% similar to the L. pneumophila Paris FeoB protein (data not shown). ClustalW alignment of feoB Fur Box and E. coli Fur box consensus sequences showed that the sequences were 68% identical and that the feoB Fur box was 74% identical to frg1 putative Fur box (Fig. 3).

In addition, a ferripyochelin binding protein (encoded by CBUA0035) was annotated on the OpH1 plasmid. However, this ORF contains a frameshift mutation, which predicts a nonfunctional protein. One other putative siderophore gene was identified, a homologue to L. pneumophila frgA, a gene with homology to aerobactin synthetase (29). However, unlike L. pneumophila, the C. burnetii frgA did not have a predicted Fur box, and the operon associated with nonclassical siderophore production in L. pneumophila, lbtAB, encoding a siderophore synthetase and a predicted transporter, was not annotated or found in the C. burnetii genome (1). Therefore, frgA is not a likely Fur-regulated candidate. Unexpectedly, a TonB homologue, associated with classical siderophile production, was not annotated, supporting the hypothesis that C. burnetii does not have the ability to import ferric iron through typical siderophores. Data from whole-genome sequence annotation suggest that transport of ferrous iron by feoB serves as the primary method of iron acquisition by C. burnetii.

We previously identified 18 putative Fur-regulated genes by an in silico approach (11). Briefly, the C. burnetii genome was searched using the E. coli Fur box consensus sequence and the putative Fur boxes of feoB and frg1. Nucleotides in the putative promoter regions matched at least 50% of the nucleotides in one or more of the three Fur boxes. These regions were also within at least 100 bases of the predicted start site.

Transcriptional analysis of putative Fur-regulated genes. We screened the putative Fur-regulated genes by using an axenic acid activation buffer system. Previously, this method has been used to analyze metabolically active, nonreplicating C. burnetii for protein expression (32, 33). In this system, the iron levels can be tightly controlled in comparison to the case when using infected cells incubated with deferoxamine. Another advantage is the ability to analyze bacterial response to iron without the presence of host factors.

To test iron regulation, gene transcript levels were measured under iron-replete and iron-depleted conditions. First, C. burnetii Nine Mile phase II was freshly purified from persistently infected L929 murine fibroblast cells. The bacteria were incubated in acid activation buffer at pH 4.5 (32) for two hours in buffer alone (trace iron) or with 10 μM or 50 μM ferrous sulfate added. The transcript level of each gene with specific primers was compared with C₅₀ value of the control, com (subtracted from average C₅₀ value of each test gene to normalize) (data not shown). To demonstrate expression level change, the 10 μM samples were used for the baseline. Calculations were based on the difference between baseline and trace iron (no additional iron) or high iron levels (50 μM).

As positive controls, Fur protein levels were evaluated in the acid activation system to ensure the regulator was present. Freshly purified C. burnetii Nine Mile phase II was incubated in the acid activation buffer at pH 4.5 with trace levels of ferrous sulfate and 50 μM ferrous sulfate. The Fur protein was detected in the bacterial lysates by using a polyclonal rabbit ChFur antibody. As a control for equal loading, Com1, a constitutively expressed protein in C. burnetii, was detected using a mouse monoclonal antibody (available in our laboratory) (see Fig. S1 in the supplemental material).

As a negative control for the experimental design, we evaluated the transcriptional regulation of fur (CBU1301) by iron. As previously described, a Fur box was not predicted in the promoter region of the annotated sequence, and therefore, it was predicted not to be regulated by iron. No significant change was observed under the high-iron-concentration condition compared to the observations under the low-iron-concentration condition (Table 2).
We predicted that \( \text{feoB} \) (CBU1766), as it has been previously identified in both \( L. \) pneumoniae and \( E. \) coli as being Fur regulated, would be regulated by iron in \( C. \) burnetii (34, 52). As expected, a 3.7-fold induction of the transcript level for \( \text{feoB} \) in buffer alone was observed in comparison with the level in buffer supplemented with 50 \( \mu \text{M} \) ferrous sulfate (Table 2). This suggested that in the presence of a very low iron concentration (i.e., the trace level), the low level of corepressor allowed the induction of the gene. This concept of iron as a repressor was supported by the repression of the transcript in the presence of iron. By use of these changes as a comparison, the remaining putative Fur regulated genes were evaluated by transcriptional analysis.

The genes predicted by the FURTA were CBU0970 (\( \text{fglI} \)) and CBU1362. Similar to the case for \( \text{feoB} \), a significant 2.8-fold induction of \( \text{fglI} \) was observed with buffer alone, and no change was observed with iron (Table 2). This further supported the FURTA prediction of Fur regulation. However, unlike \( \text{fglI} \), CBU1362 transcript levels showed no significant change in either buffer alone or the presence of iron (Table 2). This suggested that CBU1362, a predicted cation efflux pump gene, is not regulated by iron and, therefore, not a likely Fur-regulated candidate. A positive result in the FURTA screen can also occur if overexpression of the protein results in a decrease in intracellular iron levels (57), and this may occur with CBU1362, as it is a predicted efflux pump.

Few of the other Fur-regulated candidates (CBU0216, CBU1106, and CBU0968) showed a significant induction of twofold or higher in buffer alone (Table 2). Interestingly, the intergenic region between CBU0968 and CBU0970 (\( \text{fglI} \)) is small, and a palindromic Fur box may be shared between the two genes. Unexpectedly, CBU1477 had a 4.5-fold induction with 50 \( \mu \text{M} \) ferrous sulfate. However, the standard deviation was large compared to the standard deviations for other members of the group, suggesting a transcriptional response other than iron regulation.

Relative expression levels of genes were also compared based on \( C_T \) values. \( C_T \) values were converted to transcript copy numbers as previously described (10). Group I indicates the lowest expression level (10 to 100 copies of transcript), and group IV represents the highest (10,000 to 100,000 copies of transcript). CBU0216 and CBU1106 are in group I, CBU0968 and CBU0970 are in group III, and \( \text{feoB} \) is located within group IV.

**Analysis of **\( C. \) burnetii** Fur box interaction.** While \( \text{feoB} \) (CBU1766), \( \text{fglI} \) (CBU0970), CBU0216, CBU1106, and CBU0968 were predicted as being transcriptionally regulated by iron, the acid activation buffer system did not evaluate Fur regulation. To indirectly assess \( C. \) burnetii Fur interaction with the putative Fur boxes, a heterologous expression system was developed by using a two-plasmid regulator/promoter system in an \( E. \) coli fur mutant (11). This method also allowed for an indirect comparison of regulatory activities of \( C. \) burnetii Fur and \( E. \) coli Fur. The pBlue-TOPO vector containing the putative Fur box region was cotransformed with a plasmid containing \( C. \) burnetii Fur (pNP104) into BN4020, an \( E. \) coli fur deletion strain. Deferoxamine, an iron chelator, was used to deplete the level of iron in the growing cultures. The plasmid pMH15 containing the full \( E. \) coli fur gene was used for a comparison, since it has been shown that \( E. \) coli Fur can interact with Fur boxes in other bacteria (18, 57). We confirmed the production of \( C. \) burnetii Fur in the system by Western blotting using polyclonal rabbit \( C. \) burnetii Fur antibody (see Fig. S2 in the supplemental material).

The insert in the pBlue-TOPO vector varied in size from clone to clone. The size was dependent on the primers designed, based on the annotated sequence. Several of the promoter regions proved difficult to clone, which could have been due to AT-rich regions in the upstream sequence. The clones were tested in three independent experiments performed in triplicate. We chose two levels of regulation by \( t \) test statistical analysis: weak regulation, predicted by a \( P \) value of \( \leq 0.01 \), and strong regulation, predicted by a \( P \) value of \( \leq 0.005 \) (Table 3).

The region containing the \( \text{feoB} \) Fur box was used as a positive control in the development of the assay (11). As predicted, it was strongly regulated by \( C. \) burnetii Fur (Table 3). It was also strongly regulated by \( E. \) coli Fur. This supported our approach of multiple identification methods to elucidate regulatory pathways.

Four other genes were predicted to be regulated by iron in the transcriptional screen: \( \text{fglI} \) (CBU0970), CBU0216, CBU1106, and CBU0968. \( \text{fglI} \) (CBU0970) was weakly regulated by \( C. \) burnetii Fur. In support of the FURTA data, it was strongly regulated by \( E. \) coli Fur (Table 3). However, the growth of the transformant containing \( C. \) burnetii Fur (pNP104 transformed into BN4020 with a pCBU970 Fur box) was inconsistent. The degree of promoter activity, as determined in Miller units, was close to background levels, which complicated the interpretation of this gene with regard to its Fur regulation. A similar issue occurred with the CBU0968 transformant containing \( C. \) burnetii Fur. However, it was also only weakly reg-

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**TABLE 2. Expression of putative Fur-regulated genes**

<table>
<thead>
<tr>
<th>Locus tag (gene)</th>
<th>Group</th>
<th>Fold change in expression for gene with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>50 ( \mu \text{M FeSO}_4 )</td>
</tr>
<tr>
<td>CBU0216</td>
<td>I</td>
<td>0.77 ± 0.32</td>
</tr>
<tr>
<td>CBU1106</td>
<td>I</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>CBU0769</td>
<td>II</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>CBU1477</td>
<td>II</td>
<td>4.5 ± 5</td>
</tr>
<tr>
<td>CBU1546</td>
<td>II</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>CBU0280</td>
<td>II</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>CBU0395</td>
<td>III</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>CBU0432</td>
<td>III</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>CBU0611</td>
<td>III</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>CBU0920</td>
<td>III</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>CBU0968</td>
<td>III</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>CBU0970 (( \text{fglI} ))</td>
<td>III</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>CBU1362</td>
<td>III</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>CBU1493</td>
<td>III</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>CBU1556</td>
<td>III</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>CBU1600</td>
<td>III</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>CBU1695</td>
<td>III</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>CBU1851</td>
<td>III</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>CBU2012</td>
<td>III</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>CBU0963</td>
<td>IV</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>CBU1766 (( \text{feoB} ))</td>
<td>IV</td>
<td>0.78 ± 0.1</td>
</tr>
<tr>
<td>CBU1301 (( \text{fur} ))</td>
<td>IV</td>
<td>1.3 ± .1</td>
</tr>
</tbody>
</table>

* Genome copy numbers were as follows: for group I, 10 to 1 ×\( 10^2 \); for group II, 1 ×\( 10^2 \) to 1 ×\( 10^3 \); for group III, 1 ×\( 10^3 \) to 1 ×\( 10^4 \); for group IV, 1 ×\( 10^4 \) to 1 ×\( 10^5 \).
ulated by *E. coli*, suggesting that of the two closely situated genes, *frgI* was the likely Fur-regulated candidate.

Neither CBU0216 nor CBU1106 was strongly regulated by either *C. burnetii* Fur or *E. coli* Fur (Table 3). This suggests that these genes may belong to an iron-regulatory pathway independent of Fur regulation. Also, as a control for the assay, some of the genes predicted not to be regulated by iron were screened in the two-plasmid system. Surprisingly, CBU0432 and CBU0769 were both weakly repressed by Fur and strongly regulated by *E. coli*. The CBU1556 transformant containing *C. burnetii* Fur (pNP104 transformed into BN4020 with a pCBU1556 Fur box) exhibited levels similar to those observed for CBU0970. It was weakly regulated by *E. coli* Fur and was not evaluated for *C. burnetii* Fur regulation. CBU0280, CBU1493, and CBU2012 were not predicted to be iron regulated, and Fur regulation was not demonstrated (Table 3).

**Replication of *C. burnetii* in the presence of deferoxamine.**

With one annotated iron acquisition system in the genome and few additional Fur-regulated genes identified in our screens, we hypothesized that iron may play a less prominent role in replication rates of *L. pneumophila* and incubated with 0 μM, 30 μM, or 50 μM deferoxamine. Samples were taken at time zero and 24 h and 48 h after deferoxamine treatment. Compared to its replication rate in the absence of treatment, the replication rates of *L. pneumophila* over 24-h and 48-h time periods after deferoxamine treatment were significantly reduced (P value ≤ 0.05) (see Fig. S4 in the supplemental material).

J774A.1 cells were infected with *C. burnetii* Nine Mile phase II RSA 493 (avirulent passage variant of RSA 439) and allowed to infect overnight. The cells were then washed to remove uninternalized bacteria and incubated in medium containing deferoxamine. Samples were taken at time zero and 24 h, 48 h, and 72 h posttreatment. A significant increase (P value ≤ 0.01) in the genome copy number within the host cell in the presence of 30 μM deferoxamine was observed at 48 h and 72 h posttreatment compared to the number for the control (Fig. 4A). A significant decrease in generation time was observed for cells in the presence of 30 μM deferoxamine between 24 h and 72 h posttreatment (G = 10 h) and the untreated controls (G = 19 h) (P value = 0.01) (Fig. 4A). Interestingly, in all three experiments, the samples treated with 50 μM deferoxamine were inconsistent in the genome copy number, suggesting a possible modest effect to the host. Therefore, in the following experiments, we used 30 μM deferoxamine as the upper limit of treatment.

Based on these results, we theorized that by decreasing the availability of iron, the level of oxidative stress was decreased in response to the corresponding decrease in the Fenton reaction products. We therefore hypothesized that in an environment with a low level of oxidative stress, a decreased availability of iron should have minimal effect on *C. burnetii* replication. To test this hypothesis, we evaluated bacterial replication in the presence of deferoxamine in cell lines with different levels of oxidative stress as determined by the amount of expressed NADPH oxidase. J774A.16 is a murine macrophage-like cell line with an increased expression of NADPH oxidase, and J774D.9 is a cell line that is unable to express NADPH oxidase.

### Table 3. Two-plasmid regulator/promoter analysis

<table>
<thead>
<tr>
<th>Locus tag (gene) or condition</th>
<th><em>C. burnetii</em></th>
<th><em>E. coli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron regulation</td>
<td>( \text{Promoter activity (Miller units)} )</td>
<td>% Repression by iron</td>
</tr>
<tr>
<td>Vector alone</td>
<td>NA</td>
<td>71 ± 10</td>
</tr>
<tr>
<td>CBU0216</td>
<td>Yes</td>
<td>952 ± 121</td>
</tr>
<tr>
<td>CBU1106</td>
<td>Yes</td>
<td>185 ± 11</td>
</tr>
<tr>
<td>CBU0769</td>
<td>No</td>
<td>512 ± 38</td>
</tr>
<tr>
<td>CBU0280</td>
<td>No</td>
<td>609 ± 47</td>
</tr>
<tr>
<td>CBU0395</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>CBU0432</td>
<td>No</td>
<td>530 ± 22</td>
</tr>
<tr>
<td>CBU0968</td>
<td>Yes</td>
<td>ND</td>
</tr>
<tr>
<td>CBU0970 (frgI)</td>
<td>Yes</td>
<td>78 ± 4</td>
</tr>
<tr>
<td>CBU1493</td>
<td>No</td>
<td>210 ± 8</td>
</tr>
<tr>
<td>CBU1556</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>CBU1695</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>CBU2012</td>
<td>No</td>
<td>253 ± 36</td>
</tr>
<tr>
<td>CBU1766 (feoB)</td>
<td>Yes</td>
<td>974 ± 45</td>
</tr>
</tbody>
</table>

* a Genes with significantly upregulated expression levels are indicated in boldface. b P value in comparison to the value corresponding to the presence of iron, ≤0.01; c P value in comparison to the value corresponding to the absence of iron, ≤0.005; ND, not determined.

As a control for the experimental design, Trypan blue exclusion was used to assess viability of the cells in the presence of deferoxamine. Live cells were counted at 0 h, 24 h, 48 h, and 72 h posttreatment (see Fig. S3A in the supplemental material). While cell viability remained above 80% at all concentrations, it was noted that replication appeared to slow between 48 to 72 h posttreatment with 100 μM deferoxamine. This observation supports the data reported by Howe and Mallavia (33). Based on these results, we used 50 μM deferoxamine as an upper limit for treatment.

As another control for the experimental design, J774A.1 cells were infected with *L. pneumophila*, since it had been previously demonstrated that *L. pneumophila* is dependent on iron for growth (12). J774A.1 murine macrophage-like cells were infected with *L. pneumophila* and incubated with 0 μM, 30 μM, or 50 μM deferoxamine. Samples were taken at time zero and 24 h and 48 h after deferoxamine treatment. Compared to its replication rate in the absence of treatment, the replication rates of *L. pneumophila* over 24-h and 48-h time periods after deferoxamine treatment were significantly reduced (P value ≤ 0.05) (see Fig. S4 in the supplemental material).
at 48 h in the J774A.16 control samples (with increased NADPH oxidase), the genome copy started to decrease, which indicated poor growth (Fig. 4B). At 48 h, the genome copy numbers in deferoxamine-treated J774A.16 samples continued to increase significantly ($P \leq 0.01$) (Fig. 4B). In J774D.9 samples (no clonal NADPH oxidase), a trend similar to that observed in J774A.1 cells was apparent ($P \leq 0.001$) (Fig. 4C).

Replication of *C. burnetii* in iron-overloaded and decreased-iron animal models. The increased replication of *C. burnetii* in the tissue culture infection model was intriguing and atypical of bacterial pathogenesis. To test whether this phenotype was consistent in vivo, iron-overloaded and decreased-iron animal models were used to evaluate *C. burnetii* infection in these environments with different iron levels. In the iron-overloaded models of infection, an increased bacterial load has been demonstrated in other bacteria, such as *Salmonella enterica* serovar *Typhimurium* (54), *Mycobacterium tuberculosis* (38), and *Vibrio vulnificus* (63). We hypothesized that, unlike most bacteria, in the decreased-iron model, an enhanced infection would be observed, and no significant changes in infection would be observed in the iron overload model. We injected BALB/c mice with either iron dextran or saline three times a week for two weeks prior to infection. Iron dextran has been shown to be nontoxic to mice, unlike iron sulfate, ferric ammonium citrate, and iron sorbital citrate (31). After infection with *C. burnetii* Nine Mile RSA 439, the mice did not receive any further injections of iron. *C. burnetii* infection was evaluated by splenomegaly (64) and quantification of bacterial growth by enumerating genome copy number (3, 10).

BALB/c mice were infected with a high dose ($10^7$) of *C. burnetii* Nine Mile phase I, and samples were taken on day 14 postinfection. For a control, we measured the serum iron levels of the mice on day 14 postinfection. The iron-injected group had a 41% increase ($P = 0.0008$) in serum iron levels compared to the levels for the normal saline-injected group of infected mice. A similar increase was observed for control mice. We also evaluated the iron loads in the spleen and liver visually by using Prussian blue stain, which stains ferric iron (56). There was a readily observable increase in the amounts of staining between the saline- and iron-injected mice in both livers and spleens (see Fig. S5 and S6 in the supplemental material). Of note, it appeared that the iron was concentrated in granuloma formations in the livers of infected mice, possibly due to the increased prevalence of macrophages (see Fig. S6 in the supplemental material). The iron-injected group of infected mice had no significant increase in splenomegaly ($P = 0.73$) compared to saline-injected group of infected mice (Fig. 5A). However, there was a significant decrease in the genome copy number ($P = 0.02$) of the iron-overloaded group compared to the value for the control (Fig. 5B).

We then evaluated *C. burnetii* infection with lower nutritionally regulated levels of iron. In a previous study in 1999, Gomes et al. compared the efficacies associated with using an iron chelator, deferoxamine, and with a low-iron diet to develop the iron-deficient mouse model of infection (23). In a C57BL/6 mouse on a low-iron diet, serum iron levels were decreased compared to control levels, and replication of *M. avium* also decreased. In the deferoxamine-treated C57BL/6 animals, the serum iron levels were not lower than control levels and had no

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**FIG. 4.** *C. burnetii*-infected cells treated with deferoxamine. Cell lines were infected with *C. burnetii* phase II at a multiplicity of infection of 50:1. After being allowed to adhere overnight, cells were washed and treated with deferoxamine. Samples were taken at time zero, 24 h, 48 h, and 72 h. The data are representative of the trend observed in three independent experiments performed in triplicate. *C. burnetii* growth was assessed by genome copy number. (A) J774A.1 cells (the asterisk indicates a $P$ value of $\leq 0.01$). (B) J774A.16 cells (the asterisk indicates a $P$ value of $\leq 0.01$). (C) J774D.9 cells (the asterisk indicates a $P$ value of $\leq 0.001$).

(14, 22). Trypan blue exclusion was used to evaluate viability of J774A.16 and J774D.9 cells in the presence of deferoxamine. Viability remained above 85% for both cell lines under all conditions (see Fig. S3B and C in the supplemental material).

J774A.16 and J774D.9 cells were infected with *C. burnetii* Nine Mile phase II as in the J774A.1 experiment. As predicted,
effect on the bacterial growth rate, indicating that for a long-term experiment, a low-iron diet would be more effective in lowering iron concentrations (23).

As in the iron overload model, we infected BALB/c mice with a high dose (10^7) of C. burnetii Nine Mile phase I. Mice were fed a low-iron diet and deionized water for two weeks prior to infection and throughout the duration of the infection. As a control, the serum iron levels were measured at day 14 postinfection. The low-iron, infected group had a 39% decrease (P value /H11005 /0.005) in serum iron levels compared to those of the infected group on a normal diet. The control mice had serum iron levels similar to those of the low-iron, infected group. With the Prussian blue stain, there were low observable levels of iron detected in both the spleen and the liver of the mice with decreased-iron diet (see Fig. S7 and S8 in the supplemental material). Granuloma formation was observed in the livers of infected mice, but the increase in iron was not as apparent as in the iron-overloaded animal model. On day 14 postinfection, the infected mice on the low-iron diet had a significant increase in splenomegaly (P value = 0.0007) compared to that of the controls (Fig. 6A). However, there was no significant difference (P value = 0.87) between the genome copy numbers of the mice on the low-iron diet and those on the normal diet (Fig. 6B).

**DISCUSSION**

In this study, we describe the identification and initial characterization of C. burnetii fur, the genes comprising the C. burnetii Fur regulon, and a limited role for iron during C. burnetii pathogenesis. The TIGR annotation of C. burnetii Nine Mile phase I predicted a fur homologue. Based on sequence analysis of the entire fur gene, a close similarity between L. pneumophila fur and C. burnetii fur was demonstrated. A challenge in analyzing gene regulation in C. burnetii is the inability to isolate isogenic mutants. For this reason, we chose to use an E. coli fur deletion strain (RRJC-1) to evaluate the functionality of C. burnetii Fur. Within this heterologous system, C. burnetii Fur, in the presence of iron, repressed the E. coli Fur-regulated fhuF::lacZ fusion. This repression was reversed with iron chelation, indicating that the C. burnetii Fur can function as a Fur-like regulatory protein.

A regulatory difference was observed in the interaction between C. burnetii fur and the E. coli Fur-regulated fhuF::lacZ fusion compared to E. coli fur (Fig. 2). This was supported by observations of E. coli fur and C. burnetii fur in the indirect
evaluation of Fur-Fur box interaction in the two plasmid reg-
ulator/promoter heterologous system (Table 3). ClustalW
analysis of the amino acid sequences from C. burnetii and E.
coli did not reveal any significant amino acid differences in the
predicted N-terminal DNA-binding domain but did identify
differences in the C-terminal dimerization domain which may
affect function (Fig. 1). Within the predicted metal binding
motif HHXHX\_X,C (H87 to C95), the glycine residue at the
end of the motif in E. coli is replaced with a serine residue in
C. burnetii (Fig. 1) (47). It is interesting that the glycine is also
conserved in L. pneumophila fur. This motif is also predicted to
be the iron-regulatory binding site, and this change in residues
may be responsible for the differences in regulation observed
with C. burnetii Fur (50).

Demonstration of a functional C. burnetii Fur suggested that
a Fur regulon was present in the obligate intracellular patho-
gen. In some gram-negative bacteria, the Fur protein is auto-
regulated, and a Fur box has been identified in the promoter
region of the fur gene (15, 16). In addition to autoregulation,
Fur homologues have been described as being regulated, either
directly or indirectly, by transcriptional regulators such as
OxyR and RpoS (36, 65). Sequence analysis of C. burnetii fur
did not predict a Fur box in its upstream region. To test
whether production of Fur is regulated by iron in C. burnetii,
we examined gene expression at the transcriptional level. The
transcriptional profile did not demonstrate any significant ex-
pression-level changes, suggesting that transcription of C. burn-
etii Fur is not responsive to environmental iron levels.

We then identified putative members of the C. burnetii Fur
regulon. Initially, the FURTA was used to identify Fur boxes in
a C. burnetii genomic library. One limitation of this approach is
the use of E. coli Fur to identify putative C. burnetii Fur boxes.
However, this method has been used successfully for other
gram-negative bacteria as an initial screen (7, 51, 61) and for
Chlamydia trachomatis (51). Also, C. trachomatis has a more
divergent Fur, which shares 37% sequence similarity with the
E. coli protein, compared to the 77% similarity that the C. burnetii
protein shares with the E. coli protein (51). Unexpect-
edly, only two genes (CBU0970 and CBU1362) were predicted
in the FURTA screen. Sequence analysis of CBU1362 re-
vealed similarity to a family of cation efflux proteins (CDF
proteins). However, transcriptional analysis did not predict
iron regulation (Table 2). Since genes whose expression leads
to a decrease in intracellular iron levels can show positive
results in the FURTA assay (57), we theorized that CBU1362 is
involved in iron efflux. Interestingly, Grass et al. recently
described a novel role for ferrous iron export involving yiiP
(encoding FieF), encoding a member of the CDF protein fam-
ily, in E. coli (24). Since the acidity of the phagolysosome
would favor the formation of ferrous iron over ferric iron, we
hypothesized that CBU1362 would work in concert with iron
acquisition proteins to modulate the level of intracellular fer-
rrous iron.

CBU0970, designated frgl (fur-regulated gene 1), was also
identified in the FURTA screen. Sequence analysis predicted a
Fur box in the frgl promoter region with a 74% identity to the
E. coli Fur box consensus sequence. However, BLAST analysis
did not predict significant homology of the deduced amino acid
sequence to any protein in the database. We theorize that Frgl
may play a role in extracellular iron binding due to a predicted
signal sequence, a histidine-rich region, and two putative metal
binding sequences. Since the FURTA is limited to identifying
Fur boxes that are recognized by the E. coli Fur protein, we
developed in silico approaches to identify other C. burnetii
Fur-regulated genes.

One in silico approach was a screen of the TIGR genome
annotation to identify iron acquisition homologues. The iden-
tification of a ferrous iron uptake system, feoB, is consistent
with the lifestyle of C. burnetii in that the acidic environment
would favor the formation of ferrous iron. Unexpectedly, other
than a frameshifted ferrirypochelin gene (CBU0035) and a
single member of a known nonclassical siderophore transport
system, frgA (CBU0421), no other homologues to described
iron acquisition systems were identified in the C. burnetii ge-
nome. In contrast, the genome of the closely related bacterium
L. pneumophila contains genes with homology to feoB (52),
siderophore biosynthesis genes (1, 29, 37), genes encoding
ferric reductases (49), and genes encoding a novel iron acqui-
sition system that may utilize iron-loaded peptides (62). Of
particular interest, the absence of TonB and proteins involved
in classical siderophore transport suggest that ferric iron is not
an important iron source for C. burnetii. However, the pres-
ence of a frameshifted ferrirypochelin gene (CBU0035) and
the presence of frgA (CBU0421), associated with nonclassical
siderophore (37, 58) production, suggest that at an earlier
stage of evolution, C. burnetii may have possessed acquisition
systems for ferric iron. Recent studies on the C. burnetii ge-
nome suggest that this bacterium is in relatively early stage of
genomic reduction, as an adaptation to its obligate intracellu-
lar lifestyle (55).

Despite the lack of annotated iron acquisition systems, a role
for iron in the life cycle of the bacterium is suggested by the
presence of members of the heme biosynthesis pathway (hemH
[CBU0042], hemE [CBU0275], hemF [CBU1729], hemD
[CBU2077], hemY [CBU2082], and a number of putative iron-
containing proteins, including ferrodoxins [CBU0289,
CBU0581, and CBU0598] and a superoxide dismutase contain-
ing iron [CBU1708]). Also of note is the absence of homo-
logues of the highly conserved iron storage proteins ferritin
and bacterioferritin. This suggests that C. burnetii uses another
method to store ferrous iron to prevent the generation of toxic
radicals. Perhaps, Fur also acts as a ferrous iron storage site in
C. burnetii, similar to a suggested role in other bacteria (4).

The second in silico approach involved a bioinformatic sur-
voy of the genome by using the predicted Fur boxes of two C.
burnetii genes (feoB [CBU1766] and frgA [CBU0970]) and the
E. coli Fur box consensus sequence. Transcriptional analysis of
these putative Fur-regulated genes was used to predict iron
regulation compared to regulation of C. burnetii feoB. One
limitation to this study was the incubation of C. burnetii under
axenic conditions with a limited supply of nutrients to study
changes in gene expression. However, this system did allow for
tight regulation of iron levels. Using feoB as a strongly pre-
dicted Fur-regulated gene, we identified five additional genes
(including frgA [CBU0970]) that demonstrated iron regulation.
As expected, feoB (CBU1766; ferrous iron transport) and frgA
(CBU0970; predicted iron binding protein-encoding gene)
were strongly regulated by iron.

Unexpectedly, CBU1477 had a 4.5-fold increased induction
in expression with a large standard deviation. CBU1477 was
annotated as *ahpC* (encoding alkyl hydroperoxide reductase), and it is a gene that may respond to oxidative stress. The large standard deviation in the observed induction was more likely due to a response to oxidative stress generated by the increased concentration of ferrous iron (50 μM) than due to iron regulation.

The predicted iron-regulated genes (*feoB* [CBU1766], *frg1* [CBU0970], CBU0216, CBU1106, and CBU0968) were further evaluated in a regulator/promoter two-plasmid system in concert with an *E. coli fur* deletion strain. A limitation in the interpretation of this study is the use of a heterologous system. However, this approach has been used successfully with *Vibrio anguillarum* to identify Fur-regulated genes (59). Another limitation is the use of a high-copy-number plasmid for the promoterless vector and a low-copy-number plasmid for the Fur vector. This may lead to a decreased level of Fur that may not sufficiently repress the promoters tested in this screen. However, a comparatively large repression of the *C. burnetii* Fur-regulated *feoB* gene was seen when *E. coli fur*, also on a low-copy-number plasmid, was transformed into the system as a control, suggesting that the experimental parameters used herein are adequate.

The *C. burnetii* and *E. coli* Fur regulation of the shared Fur box of CBU0970 and CBU0968 was not completely evaluated. As mentioned previously, the β-galactosidase activity expression levels were close to the background levels for the *frg1* (CBU0970) transformant. Though we predict weak Fur regulation for these genes, further direct analysis of Fur interaction with the putative Fur box via DNase footprinting or some other comparable method is needed to validate where *C. burnetii* Fur regulates these divergently transcribed genes.

Surprisingly, two of the genes predicted in the transcriptional screen (CBU0216 and CBU1106) did not demonstrate Fur regulation in the two-plasmid regulator/promoter system. These results predicted that these genes may belong to an iron regulon but are not regulated by Fur. Both CBU0216 and CBU1106 were annotated hypothetical proteins. Also, in the two-plasmid system, two genes not predicted to be iron regulated by transcriptional profiling, CBU0769, a predicted glutamine amidotransferase gene (class 1), and CBU0432, a predicted major facilitator family transporter gene, were strongly regulated by *E. coli Fur* and weakly regulated by *C. burnetii* Fur. This suggested that the transcriptional screen was limited to identifying strongly iron-regulated genes.

With only one predicted iron acquisition system (*feoB*) and few Fur-regulated genes, we hypothesized that iron may play a limited role in *C. burnetii* pathogenesis, compared to its closest relative *L. pneumophila* (12). To evaluate the role of iron in *C. burnetii* replication and, therefore, survival, J774A.1 murine macrophage-like cells were infected with *C. burnetii* Nine Mile phase II and then treated with an iron chelator, deferoxamine. Deferoxamine, a hydrophilic chelator, has been previously demonstrated to track to the lysosomal compartment (48) and used in experiments with J774A.1 cells (35). The observation of an increased replication rate of *C. burnetii* in the presence of an iron chelator was intriguing and differed from results reported by Howe and Mallavia (33). These results were supported in a preliminary L929 murine fibroblast cell line experiment (unpublished data). In addition, a preliminary experiment with the addition of iron to infected J774A.1 cells, the bacterial replication decreased (unpublished data). However, one limitation of the experiment by Howe and Mallavia was microscopic quantification of *C. burnetii* infection. Since that publication, a real-time PCR method to quantify the number of genome copies of bacteria during infection has been developed (10). Also, Howe and Mallavia noted that the uninfected J774A.1 cell line at 100 μM deferoxamine at 24 h had fewer cells than the cells incubated with 20 μM deferoxamine or left untreated (33), suggesting that 100 μM deferoxamine may have had a toxic effect on the host cell. Since the only change in *C. burnetii* replication was observed in the presence of 100 μM deferoxamine in that study, this result suggests that the reduced viability of the host cell mediated the reduced infection level and was not a consequence of iron-restricted bacterial replication.

The increase in replication rate under iron-chelated conditions suggests that *C. burnetii* prefers a low-iron environment. The paucity of potential iron acquisition and storage systems, compared to the amount in *L. pneumophila*, supports this notion. We speculate that the lack of high-affinity iron acquisition systems (i.e., TonB-dependent uptake systems) is the result of selection to maintain a low-intracellular-iron environment and also suggests that chelation of iron by the host, as a primary role in defense, would not be effective in controlling *C. burnetii* infection.

Another mechanism by which iron may contribute to host defense is through the generation of toxic oxygen radicals via the oxidative burst generated by gamma interferon-activated macrophages. The oxidative burst is generated via NADPH oxidase and consists of reactive oxygen intermediates, including superoxide anion and hydrogen peroxide (44). Once hydrogen peroxide is available, ferrous iron interacts with it in the Fenton reaction to produce the highly damaging hydroxyl radicals (44). Previously, Brennan et al. demonstrated that in NADPH oxidase-negative mice infected with *C. burnetii* phase I, splenomegaly was increased significantly compared to that in the control, suggesting that oxidative stress plays a role in controlling infection (9).

When we evaluated *C. burnetii* replication in iron-chelated cells containing different levels of expression of NADPH oxidase and, therefore, oxidative stress, replication increased in a decreased iron environment. Replication also increased in the J774D.9 cell line, which lacks clonal NADPH oxidase expression. This indicates that *C. burnetii* is also sensitive to other sources of iron-generated oxidative stress, such as in the aerobic environment of tissue culture.

With a significant increase in *C. burnetii* replication in the iron-chelated tissue culture model, bacterial infection was evaluated in the iron-overloaded and decreased-iron animal models. We postulated that the decrease in iron availability would modulate the level of oxidative stress and alter host response to infection. In the decreased-iron animal model, a significant increase in splenomegaly was observed. Since splenomegaly is an indirect measure of infection, we expected to see an increase in bacterial genome copy number. However, there was no significant change. In the iron overload model, as predicted, no significant difference in splenomegaly was observed. However, there was a significant decrease in genome copy number. This suggests the intriguing concept that in the iron-overloaded animal, the host may have an increased ability to control infection. A future experiment could be the determination
of the absolute level of iron required for C. burnetii survival. Since it would be difficult to decrease iron levels in an animal model, this nutrient requirement could be further evaluated in the acid activation buffer system. In this system, the iron levels can be tightly regulated, and metabolic processes could be assessed long-term in an extremely low-iron environment.

The lack of ferric transporters and other high-affinity iron acquisition homologues, in combination with an increased replication rate of C. burnetii in the presence of an iron chelator, predicts a preference for a decreased-iron environment. We predict that iron acquisition is less of a critical pathway for C. burnetii than it is for other pathogens and that a significant phenotype would not be observed in a fur knockout mutant. Therefore, C. burnetii has a functional Fur regulon with a limited set of genes, suggesting a minimal number of iron uptake systems and a low-iron environment enhance pathogenesis of the bacterium.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant AI037744 from the National Institute of Allergy and Infectious Diseases. We thank Paul Beare for his assistance with the RNA extraction protocol. We thank Jeff Ciriolo for the kind gift of the Legionella pneumophila AA100. We also thank Jeff Ciriolo, Laura Hendrix, Renee Tsolis, and Jon Skare for their critical review of the manuscript.

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Editor: R. P. Morrison